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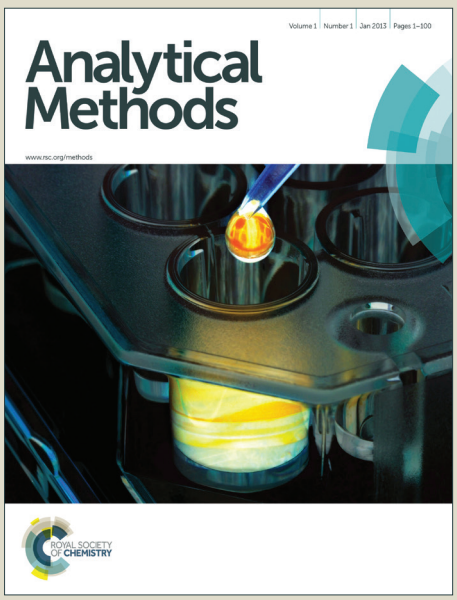

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ARTICLE

Magnetic bead-based electrochemical detection of interaction between epigallocatechin-3-gallate and STAT proteins

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In this report, the interaction of the signal transducer and activator of transcription (STAT3 and STAT5) proteins with a green tea polyphenol epigallocatechin-3-gallate (EGCG) was investigated using differential pulse voltammetry (DPV) at carbon paste electrodes (CPEs). Superparamagnetic agarose nickel beads were modified with His-tagged STAT proteins and exposed to EGCG in solution. After magnetic separation of the beads, the electrochemical oxidation of the remaining EGCG in the supernatant was monitored at ~0.18 V (vs. Ag/AgCl). The changes in the peak current signal displayed the interaction of EGCG with STAT proteins. Our electrochemical results were in agreement with the surface plasmon resonance (SPR) method that indicated the K_D values of EGCG for STAT3 and STAT5 proteins were 19.33 ± 2.11 µM and 19.53 ± 2.37 µM, respectively. To the best of our knowledge, the electrochemical detection of interaction between STAT proteins and EGCG is reported here for the first time. The voltammetric method described here provides a promising platform for the rapid and cost-effective screening of small electro-active molecules that interact with STAT proteins and other clinically important proteins.

Introduction

The signal transducer and activator of transcription (STAT) proteins are a family of macromolecules, which play an important role in gene expression, embryonic development, cell growth, cell proliferation, apoptosis and other important biological processes [1-3]. Due to their effect on cytokines and growth factors, it has been reported that there is an over expression of STAT proteins in various types of cancers such as breast, prostate, liver and pancreatic cancer [4, 5]. Recently, a significant amount of interest has been focused on finding potent inhibitors of STAT proteins, such as Inhibitor-31, which was found to bind to STAT3 at nanomolar concentrations (K_D = 300 nM) [6]. Inhibitor-31 was reported to be capable of killing glioblastoma brain cancer cells and preventing further phosphorylation of remaining STAT3 proteins present [6]. Out of the seven STAT proteins, STAT3 and STAT5 are the two most common of the family to show abnormal production in many cancers [7]. STAT5 has recently been reported to be playing a significant role in the progression of prostate cancer and acute myeloid leukemia [8].

Epigallocatechin-3-gallate (EGCG) is the major polyphenol found in green tea [9-11]. Due to its strong antioxidant activity, EGCG has been thought to aid in prevention and treatment of neurodegenerative diseases, as well as various cancers [10-14]. Thangapazham *et al.* [15] have recently reported that EGCG prevents the proliferation of breast cancer *in vitro* and *in vivo*. Our previous studies included electrochemical and surface plasmon resonance (SPR)-based analysis of EGCG with amyloid-β peptide, which is a major biomarker of Alzheimer's disease. EGCG was found to disrupt the aggregation of amyloid-β peptide and led to the production of off-pathway non-toxic oligomers [16, 17]. Similarly, EGCG was also found to disrupt the aggregation of α-synuclein protein, which is a major biomarker of Parkinson's disease [18, 19]. With regards to EGCG implication in cancer, Wang *et al.* [1] has recently reported that following treatment of the hepatocellular carcinoma cell lines with EGCG, cell proliferation was significantly suppressed, apoptosis was induced, and the expression levels of phosphorylated STAT3 proteins were dramatically lowered. Docking experiments indicated that EGCG had a strong interaction with Arg-609, one of the key residues in the STAT3-SH2 domain that contributes greatly to STAT3 and phosphorylated peptide binding [1]. Previous research aimed to study interactions between STAT3 and EGCG using SPR-based assay to determine the K_D value [1].

Though both STAT3 and STAT5 have been determined to be over expressed in many types of cancers, unlike STAT3, STAT5 has not yet been reported to interact with EGCG.

Hence, the interaction of STAT5 with EGCG is the focus of this paper. The method relied on magnetic bead-based separation techniques adapted from Palecek's influential review describing the important applications of magnetic beads in electrochemical biosensing [20]. Here, His-tagged STAT3 and STAT5 proteins were immobilized on magnetic nickel-coated agarose beads, placed in a solution of EGCG for incubation, and afterwards removed from the solution. The interactions between EGCG and STAT proteins were first investigated using differential pulse voltammetry (DPV) at carbon paste electrodes (CPEs) by monitoring the changes in the electrochemical oxidation signal of EGCG at ~0.18 V (vs. Ag/AgCl) before and after STAT3/5 exposure. SPR-based affinity binding assays between EGCG and STAT3/5 proteins were also performed to confirm the electrochemical results.

Materials and Methods

Chemicals and reagents

His-tagged STAT3 and STAT5 proteins were purchased from SignalChem (Richmond, BC). For control experiments, His-tagged Legionella collagen-like (Lcl) proteins were kindly donated by Professor Mauricio Terebiznik in the Department of Biological Sciences, University of Toronto Scarborough (Toronto, ON). His-Select nickel magnetic agarose beads and EGCG (95%) were purchased from Sigma-Aldrich (Oakville, ON). Wash buffer for the magnetic beads was prepared using 50 mM sodium phosphate solution (pH 8.0) with 10 mM imidazole and 300 mM NaCl. An elution buffer for the beads was also prepared using 250 mM imidazole in 50 mM sodium phosphate solution (pH 8.0) with 300 mM NaCl. EGCG and protein samples were prepared using 50 mM phosphate buffer solution (PBS, pH 7.4) with 100 mM NaCl. All other chemicals were of analytical grade and used as purchased.

Procedure

An aliquot (2.5 µL) of magnetic beads was diluted using 22.5 µL of PBS and added to 25 µL of 0.5 µM STAT3/5 in PBS in an Eppendorf tube. The solution was shaken at 200 rpm for 30 min. The beads were separated from the solution using a magnetic separator and were washed stringently with 20 µL aliquots of wash buffer three times for 10 min each. Then, EGCG solution was added to the beads and shaken at 200 rpm for 30 min. After incubation with EGCG, the magnetic separator was used to remove the supernatant, which was transferred into a cell for electrochemical measurements. The described procedure was also performed using Lcl proteins as control experiments. Lcl displayed no binding affinity to EGCG as confirmed by our SPR data that can be found in the Supplementary Information (Fig. S1).

Differential Pulse Voltammetry (DPV)

DPV measurements were conducted using a µAutolab type III potentiostat (Metrohm, Switzerland) and analyzed with the General Purpose Electrochemistry Software (GPES). Measurements were done using a BASi MF-2010 carbon paste electrode (Lafayette, IN), Pt-counter electrode and a Ag/AgCl reference electrode. Measurements were performed at a step potential of 0.005 V and a modulation amplitude of 0.025 V from 0 V to 0.4 V (vs. Ag/AgCl). Raw voltammograms were treated using Savitzky–Golay smoothing and baseline-correction with a moving average peak width of 0.004 V using GPES.

Surface Plasmon Resonance (SPR)

SPR measurements were performed using a Biacore X100 instrument with a Series S NTA (nitrolotriacetic acid) sensorchip. Experiments were conducted using the following solutions that were sterile filtered (0.2 µm). SPR running buffer contained 0.01 M HEPES (pH 7.4) using 0.15 M NaCl, 0.05 mM EDTA, and 0.05% surfactant P20. Nickel solution contained 0.5 mM $NiCl_2$ in running buffer. Regeneration solution contained 0.01 M HEPES (pH 8.3) with 0.15 M NaCl, 0.35 M EDTA, and 0.05% surfactant P20. Two flow cells of the sensorchip were used, one (reference flow cell) to detect nonspecific binding and background subtraction for the other (detection flow cell), which had the immobilized STAT protein on its surface. The system was washed extensively with regeneration buffer for 180 s at 10 µL/min, followed by a wash with running buffer at 30 µL/min until the baseline was stable. The nickel solution was injected to both flow cells at 10 µL/min for 60 s to form the NTA-Ni^{2+} complexes on the chip surface. Then, His-tagged STAT3/5 proteins (0.10 µM) in running buffer were immobilized on the chip at 10 µL/min for 18 min. EGCG solutions at four different concentrations were exposed to both flow cells at 30 µL/min for 2 min. All concentrations of EGCG were measured in triplicates with blank buffer solutions (no EGCG) before and after each exposure. For control experiments, His-tagged Lcl proteins (0.10 µM) were immobilized on the sensorchips, and exposed to EGCG as described above. The experimental setup was automated and analyzed using the Biacore X100 Plus 2.0 and Evaluation software.

Transmission Electron Microscopy

A 4-µL sample of the magnetic beads (incubated in STAT5) was spotted on a nickel formvar mesh grid for about 1 min. The excess liquid was then blotted dry. The sample was imaged using a Hitachi H-7500 transmission electron microscope.

Results and Discussion

After modification of the NTA-Ni complex coated magnetic beads with His-tagged STAT3 or STAT5 proteins, the beads were placed in 50 µM EGCG solution in PBS (Fig. 1A). Our method included the incubation of EGCG with STAT proteins for 30 min (Fig. 1B). The EGCG-STAT3/5 complexes were then isolated by attracting the magnetic beads to a magnet (Fig. 1C) and removing the supernatant (Fig. 1D). As a significant

amount of EGCG would bind to the proteins on the beads, monitoring the changes in the current peaks provided preliminary information on the EGCG-protein interactions.

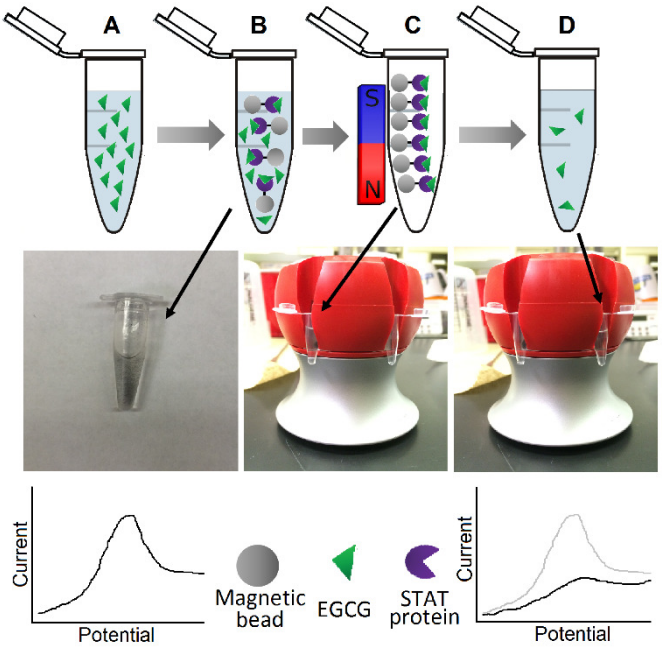

Fig. 1. Conceptual illustration of the electrochemical method for the determination of interaction between EGCG and STAT3/5 protein-modified magnetic beads as well as images of the magnetic separation process. To a solution of EGCG (A), the modified magnetic beads were added (B). The magnetic beads were attracted to a magnet (C), and the supernatant was removed for electrochemical measurements. After the binding between proteins and EGCG, the electrochemical oxidation signal of EGCG left in the supernatant (D) decreased significantly in comparison with the signal detected using the initial EGCG solution.

As shown in Fig. 2A and 2B, the electrochemical oxidation of galloyl group in EGCG was observed at ~0.15 V (vs. Ag/AgCl) in PBS (pH 7.4). Novak *et al*. [21] reported a similar peak at approximately 0.17 V (vs. Ag/AgCl) in KNO_3 supporting electrolyte solution (pH 6.0) using square-wave voltammetry at a glassy carbon electrode. Slight variation in the range of peak positions was attributed to the variations in pH of stock and wash buffer solutions. The oxidation of the galloyl group occurring at this peak has previously been found to be highly dependent on pH [21, 22]. In this paper, we chose to use differential pulse voltammetry as this is an electrochemical technique with low detection limits and the results are reproducible [23]. The average current measured for the initial 50 µM EGCG was about 206 ± 6 nA (Fig. 2C). After exposure to magnetic bead-anchored STAT3 proteins, the current signal measured was about 47% less than the initial one. Incubation of EGCG with STAT5 proteins also resulted in about 45% drop in the current in comparison with the initial signal (Fig. 2C). This result suggested that STAT3 and STAT5 proteins might have similar affinity to EGCG. To assure that the changes in electrochemical signals were due to the binding of EGCG to the STAT proteins, control experiments were conducted by modifying the magnetic beads with Lcl proteins. Once the magnetic beads were modified with Lcl proteins, they were incubated in the EGCG solution. As seen in the Supplementary Information (Fig. S1), SPR reported no affinity binding between Lcl and EGCG, therefore the electrochemical oxidation signal before and after separation were within their respective standard errors (Fig. 2C). In the absence of STAT3/5, the change in the electrochemical oxidation of EGCG after magnetic separation was negligible and we could thus conclude that STAT3/5 were indeed removing EGCG from solution by specific binding interaction (Fig. 2C). As directed by the manufacturer, the magnetic beads may be regenerated by eluting bound protein using a high concentration imidazole wash buffer. Though this was a possibility, we chose not to reuse the magnetic beads as they were relatively affordable. The reusability of magnetic beads makes them an attractive tool for the cost efficient detection of biomolecular interactions.

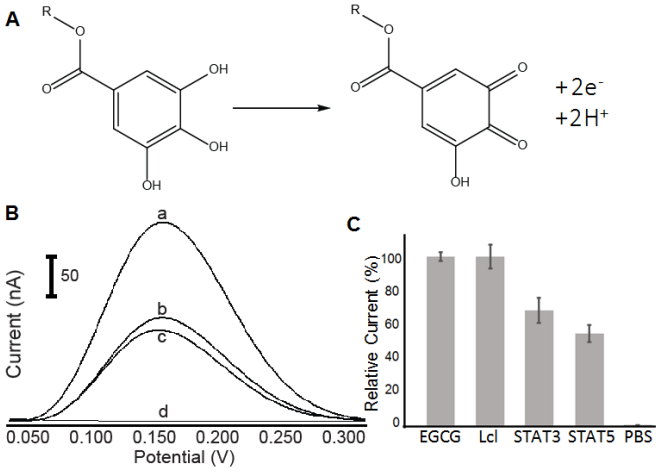

Fig. 2. (A) Oxidation of the galloyl group on EGCG. (B) Differential pulse voltammograms of 50 µM EGCG before (a) and after interaction with STAT5 (b) and STAT3-modified magnetic beads (c). (C) Summarized data as bar graphs with corresponding error bars (n=3). Other conditions were as described in the Experimental section.

SPR measurements were performed to confirm our electrochemical data. Wang *et al.* [1] have recently reported an indirect assay to detect EGCG-STAT3 protein interactions using SPR. They immobilized an EGFR-derived dodecapeptide based on the sequence surrounding the STAT3 SH2 domain Y1068 on a chip and exposed the peptides to different STAT3 samples that were incubated with various concentrations of EGCG [1]. EGCG was reported to inhibit the interaction of STAT3 with the surface-immobilized peptides with an IC_{50} value of 10-30 µM [1]. Our method aimed to directly expose the surface-immobilized STAT proteins to various

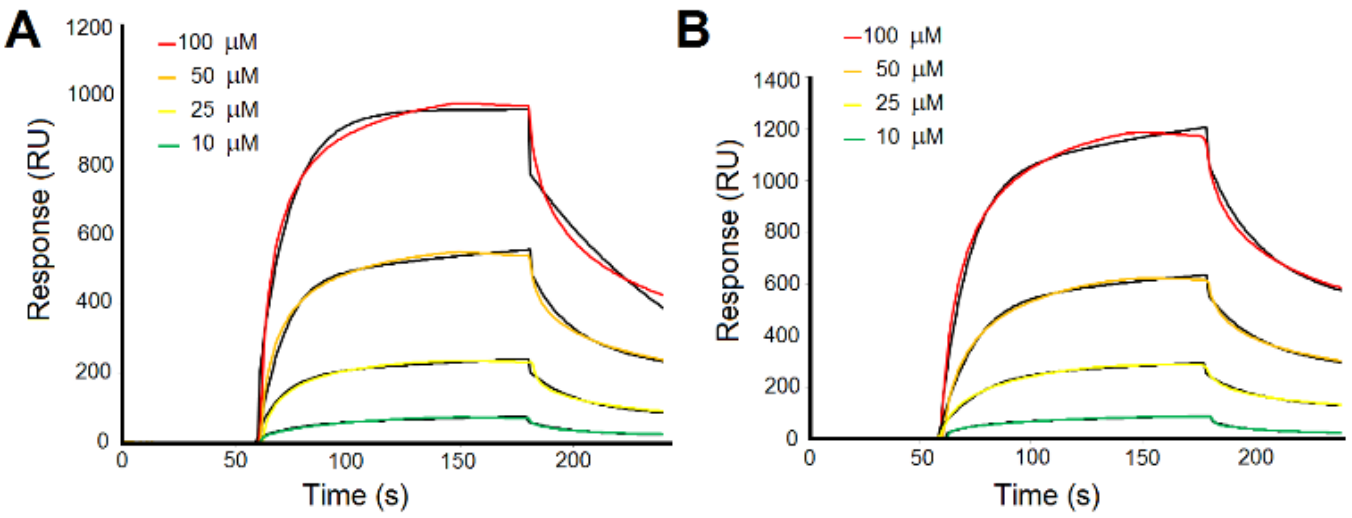

Fig. 3. Sensorgrams for the interaction between various concentrations of EGCG and 0.10 µM His-tagged STAT3 (A) and STAT5 (B) proteins immobilized on Ni2+-NTA sensorchips. The binding affinity (KD) was estimated using the Biacore X100 Evaluation software. Black lines indicate the best fit of the raw data for KD calculations. Other conditions were as described in the Experimental section.

concentrations of EGCG. Real-time sensorgrams of interaction between EGCG and STAT proteins displayed similar K_D values of 19.33 ± 2.11 µM and 19.53 ± 2.37 for STAT3 (Fig. 3A) and STAT5 (Fig. 3B), respectively. The SPR data were in agreement with the electrochemical results and supported our hypothesis that STAT5 had a similar binding affinity to EGCG as STAT3.

To visualize the surface of the agarose magnetic beads, a transmission electron microscopy image was taken and reported in Fig. S2. The mesh-like substance is thought to be the agarose casing of the beads and the dark specs within the beads were most likely impregnated paramagnetic iron which allow for the magnetic separation.

Conclusions

The electrochemical method reported here can be applied as a rapid and cost-effective screening tool aiming to find small molecules with high affinity to the STAT proteins and other clinically important proteins. The data recorded from DPV and SPR suggested a similar binding affinity of STAT3 and STAT5 with EGCG. Further studies on the inhibition of STAT3 and STAT5 using EGCG could possibly lead to the discovery of other molecules for cancer therapy. The preliminary screening of numerous natural compounds using our electrochemical method is under progress in our laboratory.

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Notes and references

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